The pharmacological profile of the vesicular monoamine transporter resembles that of multidrug transporters

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Abstract Vesicular neurotransmitter transporters function in synaptic vesicles and other subcellular organelles and they were thought to be involved only in neurotransmitter storage. Several findings have led us to test novel aspects of their function. Cells expressing a c-DNA coding for one of the rat monoamine transporters (VMAT1) become resistant to the neurotoxin *N*-methyl-4-phenylpyridinium (MPP^+) [Liu et al. (1992) Cell, 70, 539–551]. The basis of the resistance is the VMAT1-mediated transport and sequestration of the toxin into subcellular compartments. In addition, the deduced sequence of VMAT1 predicts a protein that shows a distinct homology to a class of bacterial drug resistance transporters (TEXANs) that share some substrates with mammalian multidrug resistance transporters (MDR) such as the P-glycoprotein. These findings induced us to test whether compounds that are typically transported by MDR interact also with vesicular transporters. The use of [^{3}H]reserpine binding to determine drug interactions with VMAT allowed assessment of the ability of various drugs to bind to the substrate site of the transporter. Cytotoxic compounds such as ethidium, isometamidium, tetraphenylphosphonium, rhodamine, tacrine and doxorubicin, interact specifically with vesicular monoamine transporters. Verapamil, a calcium channel blocker, is also a competitive inhibitor of transport. In the case of rhodamine, fluorescence measurements in digitonin-permeabilized cells demonstrated ATP-dependent VMAT-mediated transport. The results imply that even though the bacterial and vesicular transporters are structurally different from the P-glycoprotein, they share a similar substrate range. These findings suggest a novel possible way of protection from the effects of toxic compounds by removal to subcellular compartments.

Key words: Neurotransmitter; Parkinsonism; Neurotoxin; Synaptic vesicle; Doxorubicin; Tacrine; Verapamil; TEXAN

1. Introduction

The phenomenon of resistance of neoplastic cells and microorganisms to multiple drugs has become a serious problem in the treatment of tumors as well as of infections caused by resistant organisms [1,2]. Organisms which become cross-resistant to multiple drugs usually utilize membrane proteins that actively remove a wide variety of drugs from the cytoplasm, thus reducing their effective concentrations. The P-glycoprotein, or MDR, is the archetypal member of a continuously growing superfamily of ABC (ATP-Binding Cassette) proteins [3] or Traffic ATPases [4]. The ABC proteins pump drugs across the cell membranes in an ATP-dependent process and function in many species and cell types, from bacteria to man [3,4].

Other studies describe proteins found in certain microorganisms, which, while extruding antibiotics and toxic compounds to the medium, are mechanistically and structurally different from the Traffic ATPases and define a new family which has been called TEXANS because of their function as **T**oxin **E**xtruding **An**tiporters [5,6]. The vesicular monoamine transporters (VMAT), which remove the neurotransmitters from the cytoplasm into intracellular storage compartments show distinct homology to this new family and seem to share other mechanistic properties [6,7]. The VMATs and other members of the family actively transport substrates in exchange for hydrogen ions, utilizing thereby proton electrochemical gradients across membranes, rather than ATP [6–10].

VMATs are known for their substrate promiscuity. They transport catecholamines, indolamines, histamine, amphetamines and are inhibited by several drugs, including reserpine and tetrabenazine [6–10]. Reserpine is a potent antihypertensive alkaloid. Its therapeutic effect is based on its ability to competitively inhibit the transport of biogenic amines mediated by VMAT. Reserpine binds to the transporter with an extremely high affinity and dissociates very slowly, if at all [11,12]. Reserpine also interacts with both, MDR [13] and *Bacillus*-multidrug resistance (BMR) [14,15] albeit with affinities in the micromolar range. BMR-mutants with modified sensitivities to reserpine have been isolated and characterized [15]. The use of [^{3}H]reserpine binding measurements to determine drug interaction with the vesicular monoamine transporter allowed assessment of the relative ability of various amphetamines to bind to the substrate site of the vesicular transporter [16].

Chinese hamster ovary (CHO) fibroblasts expressing a cDNA coding for a vesicular transporter (VMAT1) become resistant to the toxic effects of the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) [7]. The basis of the protection is VMAT1-mediated transport of MPP^+ into intracellular acidic compartments, thus reducing its effective concentration. This finding, the structural similarities of VMAT and BMR and the fact that reserpine is a common inhibitor of VMAT, BMR and MDR led us to test whether VMAT shares other substrates with the multidrug transporters. In this work we show that a series of cytotoxic compounds such as ethidium, isometamidium, tetraphenyl-phosphonium, rhodamine and doxorubicin, interact specifically with vesicular monoamine transporters.

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Abbreviations: VMAT, vesicular monoamine transporter; VMAT1 and VMAT2 (previously known as CGAT and SVAT respectively) rat chromaffin granule amine transporter type 1 and 2; MPP^+, *N*-methyl-4-phenylpyridinium; BMR, *Bacillus*-multidrug resistance; MDR, multidrug resistance; CHO, chinese hamster ovary fibroblasts; TPP^+, tetraphenylphosphonium; PCA, *p*-chloroamphetamine.

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Tacrine, a long lasting anti-Che agent and verapamil, a calcium channel blocker, are also competitive inhibitors of transport. In addition, rhodamine transport has been detected in digitonin permeabilized CV-1 fibroblasts expressing either VMAT1 or VMAT2. The apparent affinity of ethidium and isometamidium to VMAT1 and VMAT2 type of transporters was compared.

2. Materials and methods

2.1. Preparation of membranes

Chromaffin granules were isolated from bovine adrenal glands by differential sedimentation as described [17]. Membrane vesicles were obtained by osmotic shock, frozen, and stored at −70°C.

2.2. ATP-dependent transport of [^{3}H]serotonin

Membranes were diluted to a protein concentration of approximately 0.075 mg/ml in a prewarmed reaction mixture (200 μl) containing 0.3 M sucrose, 10 mM K-HEPES, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM Na_2ATP, 0.4 μM [^{3}H]serotonin and the tested inhibitor in different concentrations. Initial rate of serotonin uptake was measured after incubating this suspension for 10 min at 37°C as described [17]. Competition assays were done at the indicated concentrations of inhibitor, and three concentrations of [^{3}H]serotonin, 0.05, 0.2 and 0.8 μM. Results were plotted according to Dixon plot ($1/V$ vs. [I]) and analyzed by linear regression.

2.3. [^{3}H]Reserpine binding

Membranes were diluted to a protein concentration of ≈0.15 mg/ml in a solution as described above, in presence or absence of 5 mM Na_2ATP. [^{3}H]Reserpine (20 Ci/mmol) was added to a final concentration of 1 nM. The mixture was incubated at 37°C for 10 min (when ATP was present), or 4 h (in the absence of ATP). At the indicated time the suspension was assayed as described [12], essentially a 400 μl sample was applied to a 3 ml column of Sephadex LH-20 (prepacked in a disposable syringe by centrifugation for 15 s in a clinical centrifuge), centrifuged for 1 min and the effluent was assayed for radioactivity. The assays were performed in duplicates and parallel reaction mixtures, containing 5 μM reserpine were used to subtract nonspecific binding which was typically less than 10% of the binding to membranes. All data presented are mean values of duplicates.

2.4. Growth of cells, transfections and transport assays

The protocol for functional expression of rat VMAT1 was essentially [18] as described by Erickson et al. [19]. CV1 cells were grown in DMEM supplemented with 10% FCS and 2 mM glutamine in 24-well collagen-treated plates. The cells were infected with recombinant vaccinia virus encoding bacteriophage T7 DNA polymerase [20] and after 30 min they were transfected with 2 μg of plasmid DNA coding for r-VMAT1 or b-VMAT2, using 6.4 μg transfection reagent per well (DOTAP-Boehringer). After 18–20 h cells were rinsed with uptake buffer containing 110 mM potassium tartarate, 5 mM glucose, 0.2% BSA, 200 μM $MgCl_2$, 1 mM ascorbic acid, 10 μM pargyline and 20 mM PIPES at pH 7.4. Cells were permeabilized for 10 min at 37°C in uptake buffer containing 10 μM digitonin. The medium was removed and replaced with fresh buffer without digitonin containing 5 mM $MgSO_4$, 5 mM Na_2ATP and the corresponding tracers and/or inhibitors as indicated for the specific experiments. After 10 min the reaction buffer was aspirated and discarded and the cells were washed with ice-cold uptake buffer containing 2 mM $MgSO_4$ and no tracers. The cells were then collected with 1% SDS and radioactivity assessed by liquid scintillation. All data presented are mean values of triplicates.

2.5. Assay of Rhodamine transport

CV-1 cells were grown on glass cover slips (1 × 1 cm) attached to the bottom of culture dishes (36 mm diameter, Nunc, Denmark) which were previously perforated to give a 0.6 cm diameter hole. The dishes were cleaned and rinsed with distilled water and ethanol, dried and exposed overnight to UV light, then dishes were coated with collagen as described above. Cells were infected with the virus and transfected with either r-VMAT1 or b-VMAT2 and Rhodamine 6G transport was assayed 16 to 20 h after the infection. The assay was essentially the same as [^{3}H]serotonin transport. After the digitonin permeabilization, cells were rinsed and medium was replaced with fresh buffer containing 5 mM ATP and 0.5 μM Rhodamine 6G. Fluorescence was followed by fluorescence cell imaging on a PTI deltascan station (505 nm excitation) (PTI GmbH, Wedel, Germany) connected to a Nikon Diaphot microscope (D-510 dichroic mirror, 520–560 interference emission filter), a Hamamatsu ICCD camera c2400-97 interfaced with a PTI D500 Video

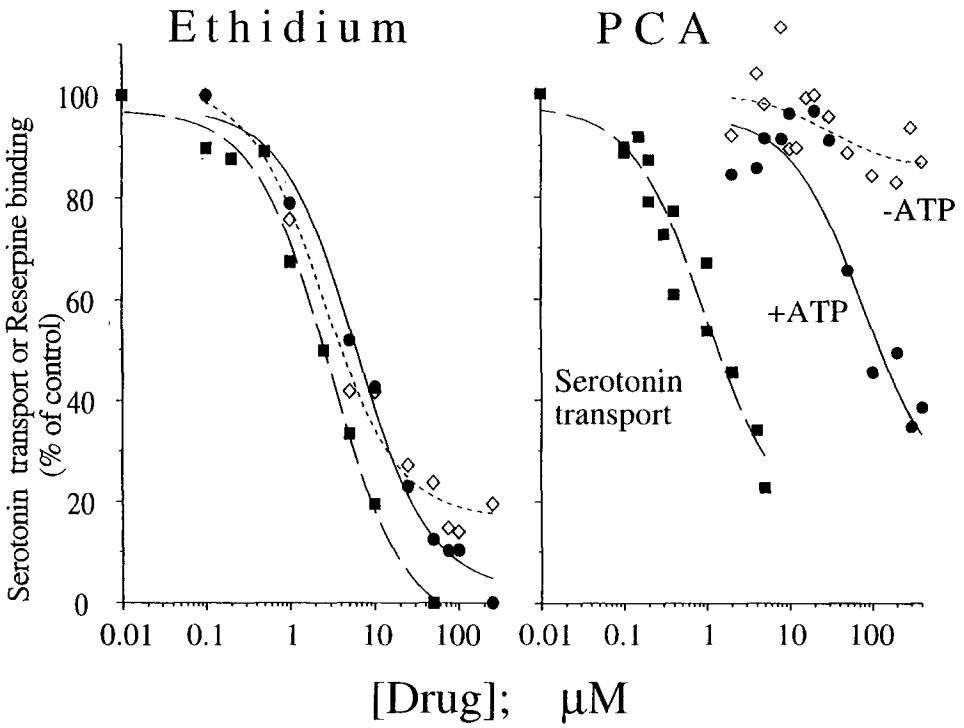

Fig. 1. Inhibition of chromaffin granule [^{3}H]serotonin transport and [^{3}H]reserpine binding by ethidium and PCA. The effect of ethidium and PCA on ATP dependent transport of [^{3}H]serotonin (■) and [^{3}H]reserpine binding in the presence (●) or in the absence (◇) of ATP was tested in section 2 at the drug concentrations indicated.

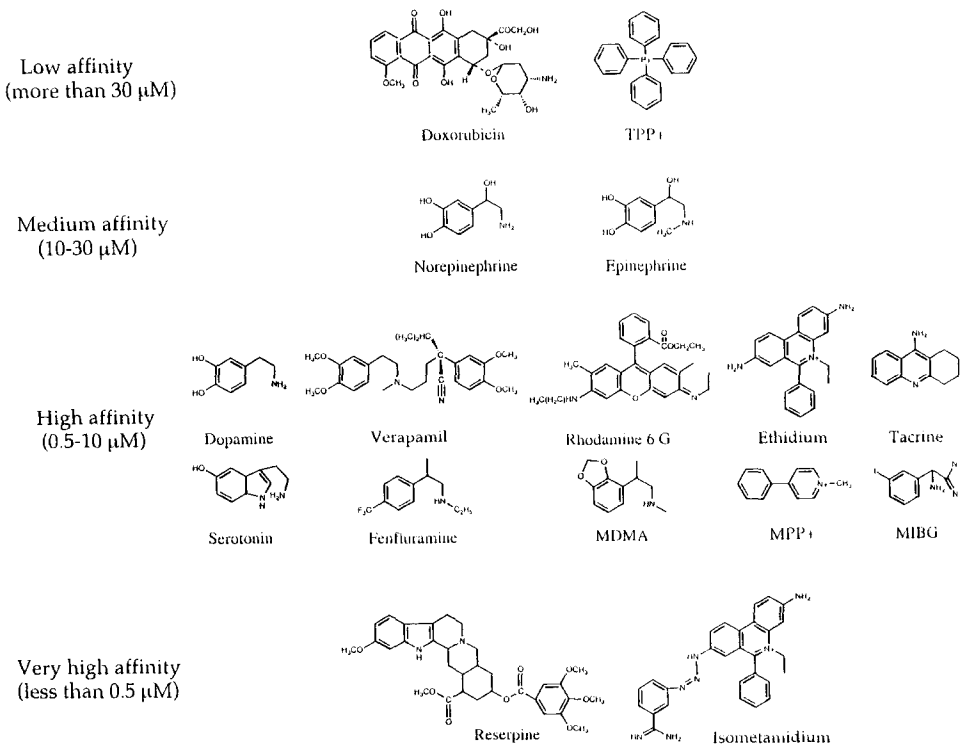

Fig. 2. Structural formula of inhibitors and substrates of vesicular monoamine transporters. The apparent affinities as determined in this work and in the references quoted in the body of the paper.

Imaging System. Images were automatically collected (at 30 s intervals) following excitation shutter opening for 2 s intervals and were stored for subsequent processing. The pseudocolor images obtained were used for fluorescence measurements (average signal of four different cell areas) at the indicated times.

3. Results

3.1. A variety of cytotoxic compounds interact with vesicular monoamine transporters

In the present study, the ability of substrates of MDR and BMR to inhibit VMAT function was tested. Previously, our understanding of solute interaction with VMAT was limited to measurements of transport and its driving force, ΔpH. In the studies presented here, the ability of a variety of solutes to interact directly with VMAT has been evaluated by measuring their effect on [^{3}H]reserpine binding. Reserpine is thought to bind directly to the substrate site of the transporter and is, therefore, a more precise indicator of interaction with that site. The strength of this approach has been shown already in a study of the interactions of various amphetamines with VMAT [16]. In the present work we find several cytotoxic compounds that inhibit [^{3}H]serotonin uptake and [^{3}H]reserpine binding to the chromaffin granule amine transporter from bovine adrenal.

The toxic organic weak base ethidium is transported by BMR [14] and interacts also with MDR [1]. It also inhibits serotonin transport into chromaffin granules membrane vesicles from bovine adrenal in a concentration-dependent manner (Fig. 1A). At this serotonin concentration (0.4 μM), half maximal inhibition is observed at 3 μM ethidium. When the experiment was repeated at three concentrations of serotonin, analysis of the data on Dixon plots revealed a competitive inhibition with an apparent K_i of 2.5 μM (not shown). Since the transport of serotonin is driven by a proton electrochemical gradient and ethidium is an hydrophobic weak base, it could potentially inhibit ATP-dependent transport by two mechanisms. Dissipation of transmembrane pH gradients by nonionic diffusion of ethidium could remove the driving force for transport. Since influx of each amine molecule requires efflux of two H^+ ions, transport is particularly sensitive to small changes in intravesicular pH. Alternatively, ethidium might interact directly with the substrate binding site to competitively inhibit transport. Since substrates for VMAT are known to compete with [^{3}H]reserpine for binding, we measured the ability of ethidium to inhibit binding of [^{3}H]reserpine to chromaffin granule membranes. In Fig. 1A we see the results of such an experiment in which reserpine binding was assayed in the presence and in the absence of ATP. ATP is known to accelerate binding of reserpine by virtue of its ability to generate a proton electrochemical gradient via the V-ATPase. Thus, again, inhibition of [^{3}H]reserpine binding could result from competition at the reserpine binding site or dissipation of ΔpH. By measuring binding rates in the presence and absence of ATP, we were able to distinguish between these two mechanisms. Half maximal inhibition of [^{3}H]reserpine binding occurred at 5 μM ethidium, whether ATP-dependent or -independent binding was measured and this is practically identical to the concentration which inhibits serotonin transport. The findings unequivocally demonstrate that ethidium exerts its inhibitory effect as a competitive inhibitor of the transporter rather than through an effect on the $\Delta\tilde{\mu}_{H^+}$.

For comparison, the mode of action of a weak base such as *p*-chloroamphetamine (PCA) is described in Fig. 1B. This sympathomimetic amine, inhibits serotonin transport through dissipation of $\Delta\tilde{\mu}_{H^+}$ ($K_{0.5}$ of 1 μM and see also [16]). PCA has no

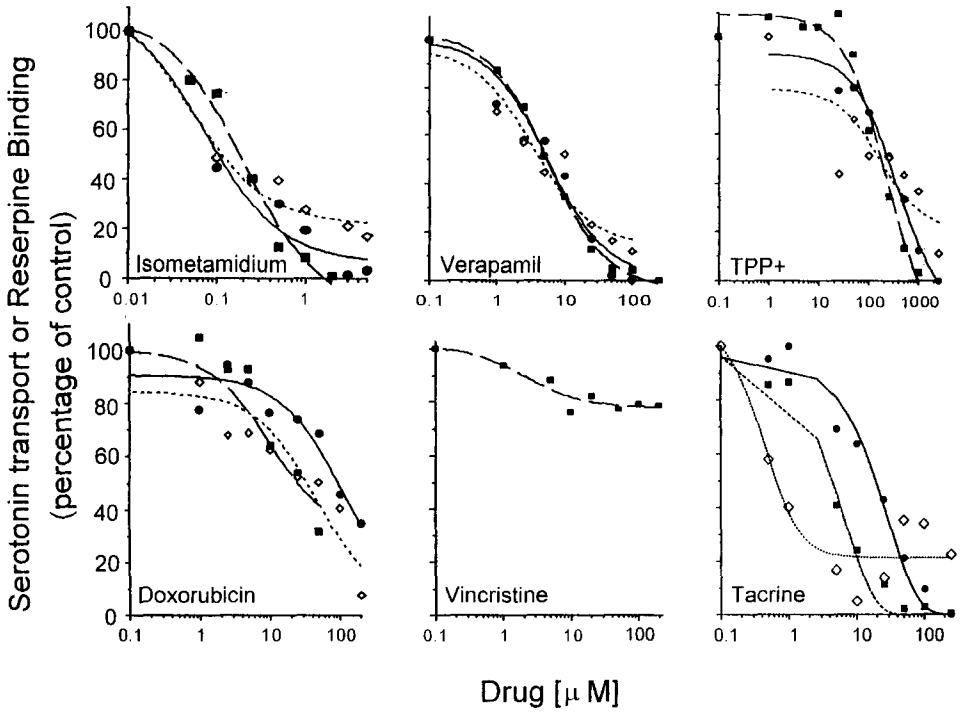

Fig. 3. Inhibition of chromaffin granule [^{3}H]serotonin transport and [^{3}H]reserpine binding by MDR substrates. The effect of the indicated compounds on ATP dependent transport of [^{3}H]serotonin (■) and [^{3}H]reserpine binding in the presence (●) or in the absence (◇) of ATP was tested to assess their ability to interact with the vesicular monoamine transporter from bovine adrenal chromaffin granules.

significant effect on reserpine binding to the transporter measured in the absence of ATP (up to 800 μM of the drug). As expected from an agent collapsing $\Delta\tilde{\mu}_{H^+}$, PCA inhibits the rate of ATP-dependent reserpine binding at intermediate concentrations, ($K_{0.5}$ = 120 μM) since this reaction is less sensitive to changes in ΔpH than transport [12,16]. Although not evident from the figure, the binding rates are inhibited to the levels in the absence of ATP.

Using the experimental paradigm described the effect of the following substrates or 'modulators' of multidrug transporters has been tested (structural formulas in Fig. 2): Verapamil is a calcium-channel blocker [21]. Tetraphenylphosphonium (TPP^+), a lipophylic cation, is widely used for measuring membrane potential [22]. Doxorubicin is an antineoplastic agent [21]. Isometamidium is an antitrypanosomal agent thus far untested in other multidrug transporters [23]. Tacrine, is a long lasting anti-Che agent [24]. All were shown to be inhibitors of serotonin transport and reserpine binding (Fig. 3). The most potent inhibitor was isometamidium, which inhibited all reactions with a $K_{0.5}$ of 0.25 μM for the three reactions tested. Verapamil inhibited with a $K_{0.5}$ of 7.5 μM, while TPP^+ and Doxorubicin were relatively poor inhibitors. TPP^+ inhibits serotonin transport with a $K_{0.5}$ of about 80 μM. TPP^+ is the only inhibitor in which the charge is in a phosphonium moiety rather than in the classical amine. Therefore a Dixon plot analysis was carried out to confirm the competitive nature of the inhibition by the latter and the apparent K_i calculated was 50 μM (not shown); inhibition of reserpine binding required slightly higher concentrations of the cation.

Some compounds with no effect on transport were also screened: Vincristine is an antineoplastic agent, substrate of MDR [1] (Fig. 3). Norfloxacin is an antibacterial analog of nalidixic acid and is the classical substrate of BMR [14]. Paraquat is an herbicide structurally related to the neurotoxin MPP^+. Acetylcholine is structurally unrelated but it is a substrate of the highly similar vesicular acetylcholine transporter (VACHT). None of the above mentioned compounds had any effect on serotonin transport up to concentrations of 200 μM (only the results for vincristine are shown in Fig. 3 as an example, the rest of the data are not shown).

Two distinct types of VMAT's have been characterized: VMAT1 and VMAT2 type [7]. The two transporters are highly similar, coded by genes in different chromosomes and have been shown to differ in some of their pharmacological properties [25]. We compared the potency of ethidium and isometamidium on the two transporter types transiently expressed in CV1 cells. Both inhibit VMAT2 with a slightly higher potency than VMAT1 (Fig. 4). The IC_{50} values are respectively 4.5 and 0.1 μM for VMAT2 and 10 and 0.3 μM for VMAT1.

3.2. *Rhodamine 6G is actively transported by both VMAT1 and VMAT2*

Competitive inhibition suggests interaction at the substrate site of the transporter. However, not all the drugs interacting at the recognition site are necessarily transported. Thus, while dopamine, adrenaline, noradrenaline, serotonin, fenfluramine, MPP^+, MBIG are substrates of VMAT, reserpine is not transported even though it shows the highest affinity for the binding site. Assaying transport of hydrophobic substrates implies problems related with their high passive leaks and non-specific binding. Therefore we selected a substrate which can be easily monitored on-line by measuring fluorescence levels on single cells. Rhodamine 6G was tested first for inhibitor of [^{3}H]serotonin transport and [^{3}H]reserpine binding as described above

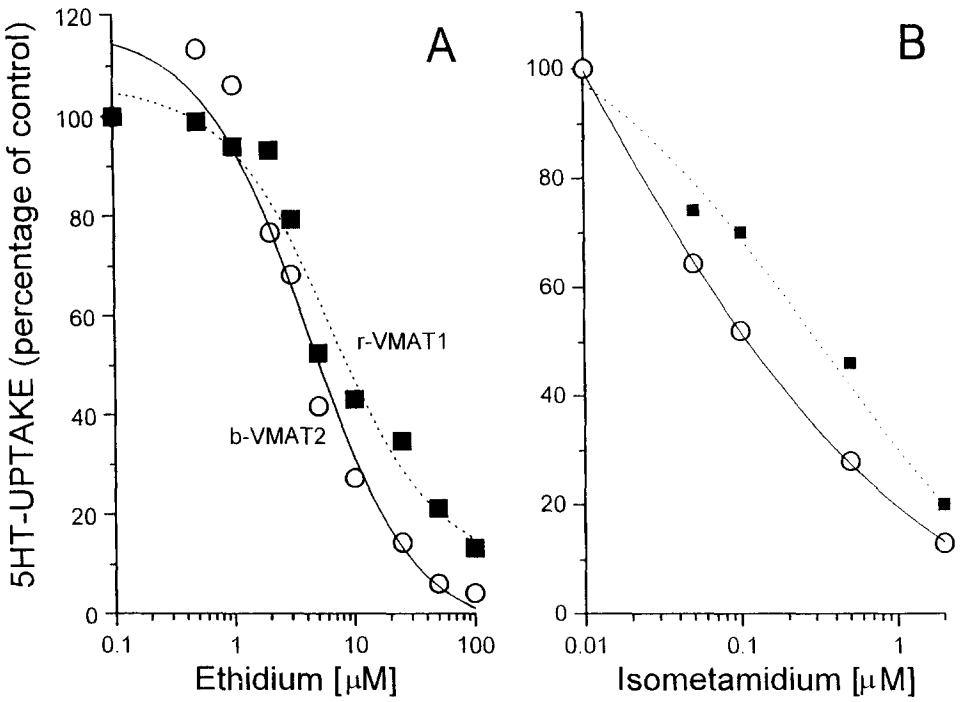

Fig. 4. Inhibition of r-VMAT1 and b-VMAT2 [^{3}H]serotonin transport by ethidium, and isometamidium. The effect of ethidium and isometamidium on ATP dependent transport of [^{3}H]serotonin was tested in CV1 cells transfected with the appropriate clones as described in section 2.

and found to inhibit both with an IC_{50} of 5 μM (Fig. 5A). Rhodamine 123 and 6G are used as mitochondrial markers and they accumulate in these organelles in an energy dependent manner [26]. Permeabilization of the cell by digitonin causes depletion of the energy sources as detected by the fact that VMAT-mediated transport into subcellular organelles dependent on externally added ATP [18,19]. Mitochondria of digitonin permeabilized CV-1 cells do not significantly accumulate Rhodamine (Fig. 5B). However when either VMAT or VMAT2 are expressed in these cells, a rapid accumulation is observed, and this process is inhibited by reserpine (5 μM) (not shown).

4. Discussion

4.1. *Vesicular monoamine transporters show a high substrate promiscuity similar to multidrug transporters*

In this work an interaction of vesicular monoamine transporters with a wide variety of compounds has been demonstrated: ethidium, isometamidium, doxorubicin, tacrine, rhodamine, tetraphenylphosphonium and verapamil (Fig. 2). Other drugs recognized by VMATs in addition to adrenaline, noradrenaline, dopamine and serotonin include histamine [25], the neurotoxin MPP^+ [7,27–29]; fenfluramine, the sympathomimetic amines and 3,4-methylenedioxy-methamphetamine (MDMA, also known as 'ecstasy') [16], and meta-iodobenzylguanidine [30], an adrenal imaging agent used for the scintigraphic detection of human pheochromocytoma. A group of inhibitors that seem to act on a site different from the substrate site and represented by tetrabenazine, ketanserine and related compounds [31] has not been included in the figure.

Interestingly, most of the compounds above mentioned have been reported to interact with other multidrug transporters suggesting that there is a marked overlap in substrate specificity in the various multidrug transporters. This may suggest a possible common solution to the problem of polyspecificity with several minor modifications. This polyspecificity appeared during evolution in five different families: in ABC type, TEXANs, Mini Texans, RND and the kidney organic cation transporter. This may hint at a common need of primitive living cells for mechanisms of protection from an unfriendly environment. Later on in evolution some of the multidrug transporters which evolved have specialized to given functions such as neurotransmitter transport, phospholipid translocation and others.

An interesting question is how these proteins can recognize with high affinity such a broad array of substrates. One such system which copes with this type of problem and has been studied at the atomic level are the Major Histocompatibility Complex (MHC) molecules [32,33]. The conclusion from these studies is that the MHC molecules can bind a variety of peptides because they interact with a few structural elements common to all peptides. Binding occurs with high affinity primarily because of extensive interaction with the peptide backbone and with the amino and carboxy termini of the peptide. The side chains that differ from peptide to peptide have little if any effect. A parallel, although mechanistically different situation occurs in cholinesterases, many of which interact with a wide array of asymmetric ligands with a cholinium moiety at one end and a charged acyl moiety at the other. The interaction is mediated through the primary attraction to negatively charged surface residues and subsequent hydrophobic interactions [34].

We speculate that similar principles are utilized by polyspecific transporters. A few generalizations can be tentatively drawn based on our studies on VMAT. All the effective known substrates and inhibitors of VMAT contain an aromatic ring and a positive charge, and therefore we can suggest that these are involved in the interaction (Fig. 2). In a given series of com-

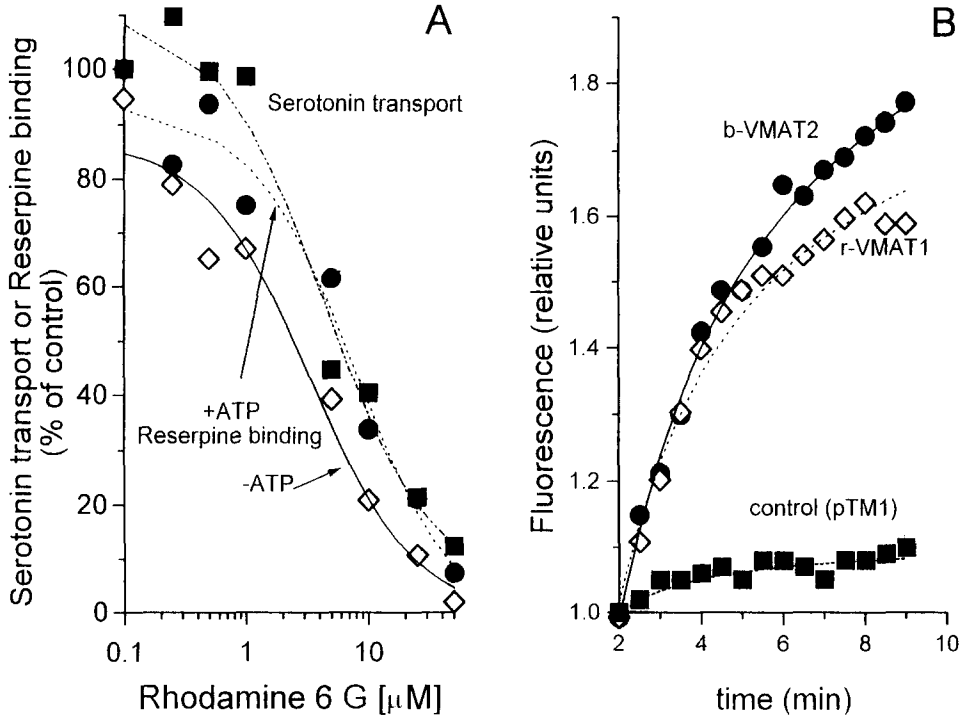

Fig. 5. ATP dependent uptake of Rhodamine 6G in CV1 cells transfected with b-VMAT2 or r-VMAT1. (A) Inhibition of [^{3}H]serotonin transport (■), and [^{3}H]reserpine binding in the presence (●) or absence (◇) of ATP. (B) Rhodamine 6G fluorescence was assessed in digitonin permeabilized cells as described in section 2.

pounds, hydroxyl, methoxy, or amino substitutents in the ring, improve the affinity of the substrates [8–10,16]. Introduction of a negative charge in the molecule greatly diminishes the affinity of interaction (see here norfloxacin and also [35,37] for derivatives of reserpine and fluoroserotonin). The only known inhibitor with a positive charge other than ammonium, is TPP^+, which displays a relatively low affinity (K_i = 50 μM). The interaction therefore seems to be mediated through a primary low affinity attraction to positively charged residues and subsequent more specific hydrophobic interactions with a properly substituted aromatic ring.

Many of the compounds described in Fig. 2 have also been documented as substrates of VMATs: mIBG [30], the neurotoxin MPP^+ [7,27–29] and Rhodamine 6G (this work). To assess whether any of the other compounds described here as inhibitors are also substrates and to test VMATs possible roles in conferring resistance to the toxic effect the resistance of CHO fibroblasts expressing VMAT has been compared to that of a non-expressing line [7]. The following compounds were tested: ethidium, isometa-midium and doxorubicin. Only marginal differences in resistance were observed in all cases (data not shown). In both cell lines reserpine dramatically increased the sensitivity to all the toxic compounds even though it was not toxic by itself. These observations are in agreement with findings that CHO cells have a high level of expression of an MDR-type protein with a specificity very similar to VMAT. In this cell line MPP^+ is the only toxic substrate specific to VMAT found thus far.

It is not known yet whether mammalian TEXANs play in living organisms any role other than transport of neurotransmitters. In microorganisms, the TEXANs have been identified based on their ability to confer resistance against antibiotics and other toxic compounds. The results presented in this report suggest that VMAT are multidrug transporters. This follows demonstration that VMATs can protect CHO fibroblasts from the toxicity of a cytotoxic agent, such as MPP^+ [7]. We can now only speculate about a possible role of these proteins and other TEXANs in the intact organism as another novel way of detoxification at the cellular level by compartmentalization.

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